Nitric Oxide–Producing Myeloid-Derived Suppressor Cells Inhibit Vascular E-Selectin Expression in Human Squamous Cell Carcinomas

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Squamous cell carcinomas (SCCs) are sun-induced skin cancers that are particularly numerous and aggressive in immunosuppressed individuals. SCCs evade immune detection at least in part by downregulating E-selectin on tumor vessels, thereby restricting entry of skin-homing T cells into tumors. We find that nitric oxide (NO) potently suppresses E-selectin expression on human endothelial cells and that SCCs are infiltrated by NO-producing iNOS$^+$ CD11b$^+$ CD33$^+$ CD11c$^-$ HLA-DR$^-$ myeloid-derived suppressor cells (MDSCs). MDSCs from SCCs produced NO, transforming growth factor-β (TGF-β), and arginase, and inhibited endothelial E-selectin expression *in vitro*. MDSCs from SCCs expressed the chemokine receptor CCR2 (chemokine (C-C motif) receptor 2) and tumors expressed the CCR2 ligand human β-defensin 3 (HBD3), suggesting that CCR2/HBD3 interactions may contribute to MDSC recruitment to SCCs. Treatment of SCCs *in vitro* with the inducible nitric oxide synthase (iNOS) inhibitor N_ω-nitro-L-arginine(L-NNA) induced E-selectin expression at levels comparable to imiquimod-treated SCCs undergoing immunologic destruction. Our results suggest that local production of NO in SCCs may impair vascular E-selectin expression. We show that MDSCs are critical producers of NO in SCCs and that NO inhibition restores vascular E-selectin expression, potentially enhancing T-cell recruitment. The iNOS inhibitors and other therapies that reduce NO production may therefore be effective in the treatment of SCCs and their premalignant precursor lesions, actinic keratoses.

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INTRODUCTION

Over 700,000 squamous cell carcinomas (SCCs) are diagnosed each year in the United States (Rogers *et al.*, 2010). The treatment of nonmelanoma skin cancers, of which SCC is the second most frequent type, account for 4.5% of all Medicare cancer costs (Berg and Otley, 2002; Housman *et al.*, 2003). Although most are curable by surgical excision, 4% metastasize to the lymph nodes and 1.5% of SCC patients die from metastatic or locally aggressive disease (Brantsch *et al.*, 2008). SCCs are a leading cause of death among organ-transplant recipients. These patients have a 65- to 250-fold increased risk of developing SCCs; nearly 10% of these cancers metastasize and the majority of patients die as a result (Berg and Otley, 2002; Euvrard *et al.*, 2003). Currently, wide surgical excision is the only treatment for invasive SCCs. In addition to the burden of invasive cancers, actinic keratoses, the premalignant precursor lesion of SCCs, are the third most frequent reason in the United States for consulting a dermatologist (Feldman *et al.*, 1998). Over 5.2 million physician visits are made each year for the treatment of actinic keratoses at a cost of over $900 million (Warino *et al.*, 2006).

Immune evasion in human SCCs appears to primarily result from aberrant T-cell homing. Vessels in SCCs lack expression of E-selectin, a skin addressin that is expressed at baseline by cutaneous postcapillary venules, is upregulated with inflammation, and by binding to cutaneous lymphocyte antigen (CLA) on skin-homing T cells, mediates the first step of T-cell recruitment into skin (Chong *et al.*, 2004; Kupper and Fuhlbrigge, 2004; Clark *et al.*, 2008). As a

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Abbreviations: APCs, antigen presenting cells; CCL2, chemokine (C-C motif) ligand 2; CCR2, chemokine (C-C motif) receptor 2; CLA, cutaneous lymphocyte antigen; DMEC, dermal microvascular endothelial cell; HBD, human β-defensin; HPF, high-power field; HUVEC, human umbilical vein endothelial cell; iNOS, inducible nitric oxide synthase; L-NNA, N_ω-nitro-L-arginine; MDSC, myeloid-derived suppressor cell; NO, nitric oxide; SCC, squamous cell carcinoma; TGF-β, transforming growth factor-β; TLR7, Toll-like receptor 7; TNF-α, tumor necrosis factor-α

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result, these tumors exclude CLA^+ skin-homing T cells, the cell type that provides cutaneous immune surveillance and would be expected to contain tumor-specific T cells (Clark, 2010).

Topical treatment of SCCs with the Toll-like receptor 7 (TLR7) agonist imiquimod induces endothelial activation, massive infiltration of tumors by CLA^+ T cells producing IFN-γ, perforin, and granzyme, tumor cell death, and histologic evidence of tumor regression (Clark *et al.*, 2008; Huang *et al.*, 2009). This rapid and effective immune response suggests that primed SCC-specific T cells exist in the circulation but these cells cannot gain access to the tumor. Imiquimod can be used to treat SCCs in poor surgical candidates (Peris *et al.*, 2006). In solid organ-transplant recipients, a course of imiquimod was effective and did not engender graft rejection in the 6 or 12 months that patients were followed (Brown *et al.*, 2005; Ulrich *et al.*, 2007) but concerns linger that repeated use of imiquimod could engender graft rejection or shorten the life of an allograft. There is therefore a need to understand why blood vessels in SCCs fail to express T-cell homing addressins such as E-selectin and to identify new agents that can induce endothelial activation and restore appropriate T-cell homing without broad, nonspecific activation of the immune system. Below, we present our findings that nitric oxide (NO) production in SCCs contributes to suppression of E-selectin expression by tumor vessels and that agents that inhibit NO production may be effective therapeutic strategies for the treatment of SCCs.

RESULTS

Vascular E-selectin expression correlates with tumor infiltration by CLA^+ T cells and histologic evidence of tumor regression

We previously observed qualitative increases in vascular E-selectin expression and T-cell infiltration after TLR7 agonist treatment of SCCs (Clark *et al.*, 2008; Huang *et al.*, 2009). To quantitatively study the relationship of vascular E-selectin expression and tumor infiltration by skin-homing CLA^+ T cells, we counted the percentage of tumor vessels expressing E-selectin and the number of infiltrating CLA^+ T cells in untreated SCCs and SCCs treated with TLR7 agonist before excision. In agreement with earlier observations, vascular E-selectin expression was absent or low in untreated tumors and markedly upregulated in SCCs treated with the TLR7 agonist imiquimod (Figure 1a and d). In untreated tumors, 6.7% of blood vessels in tumors expressed E-selectin ($n=5$, SEM 1.53), whereas 34.2% of blood vessels expressed E-selectin in tumors treated with TLR7 agonist before excision ($n=3$, SEM 3.04). The difference between treated and untreated SCCs was statistically significant ($P<0.0001$). Similarly, recruitment of CLA^+ T cells was low in untreated SCCs but greatly enhanced in treated tumors (Figure 1b-d). Untreated tumors were infiltrated by a mean 21.3 CLA^+ T cells per high-power field (HPF, $n=6$, SEM 5.91), whereas tumors treated with TLR7 agonist before excision contained a mean 256.6 CLA^+ T cells per HPF ($n=3$, SEM 47.2, $P=0.0002$). There was in fact a strong linear correlation between vascular E-selectin expression and infiltration by CLA^+ T cells (correlation coefficient $R=0.94$) and only tumors with both features had histologic evidence of tumor regression (Figure 1d).

SCCs are infiltrated by NO-producing $CD11c^-CD11b^+$ HLA-DR^- myeloid-derived suppressor cells

Cells expressing inducible nitric oxide synthase (iNOS) were prominent in untreated SCCs (Figure 2a and b; Clark *et al.*, 2008). We previously observed faint staining of $iNOS^+$ cells for CD11c, suggesting a possible dendritic cell lineage. However, follow-up staining with multiple anti-CD11c antibodies demonstrated that these cells were in fact $CD11c^-$, $CD11b^+$, and HLA-DR^-, a phenotype shared by myeloid-derived suppressor cells (MDSCs) identified in several human cancers (Figure 2c, d, and h; Filipazzi *et al.*, 2007; Diaz-Montero *et al.*, 2009; Corzo *et al.*, 2010; Gabitass *et al.*, 2011). $iNOS^+$ cells lacked expression of the macrophage marker CD163, the T-cell marker CD3, and the endothelial cell marker CD34 (Figure 2e-g) and were $CD14^-$ (data not shown). Further characterization by flow cytometry analysis of dispersed cells isolated from collagenase-treated SCCs demonstrated that $CD11b^+$ HLA-DR^- cells expressed CD33, a subset expressed iNOS, and the majority expressed transforming growth factor-β (TGF-β), a phenotype consistent with human MDSCs (Figure 3a). Real-time PCR analysis of $CD11b^+$ cells isolated by magnetic bead separation from collagenase-dispersed tumors demonstrated that $CD11b^+$ cells expressed arginase I (Figure 3b). $iNOS^+$ $CD11b^+CD11c^-$HLA-DR^- cells were present in 16/16 untreated SCCs (10 SCCs were studied by immunostaining of cryosections and 6 by flow cytometry analysis of collagenase-treated tumors). As we previously reported, $iNOS^+$ cells were not present in imiquimod-treated SCCs undergoing regression (Clark *et al.*, 2008). $CD11b^+$ HLA-DR^- cells made up a mean 6% of total tumor cells in collagenase-digested SCCs but were rare (0.8%) in normal human skin (Figure 3c). Selective gating on iNOS-expressing cells demonstrated that MDSCs were the prominent cell type expressing iNOS in most tumors, although it was also expressed by other cell types within the tumor microenvironment (Figure 3d). A mean 51% of total $iNOS^+$ cells in four collagenase-digested SCCs were $CD11b^+$ $CD11c^-$ HLA-DR^- $CD33^+$ MDSCs (SEM 10.3).

To confirm that NO was produced within the SCC tumor microenvironment, we measured nitrate and nitrite levels from tumor supernatants using the Griess method (Figure 3e). Although levels varied, significant NO production was observed in all tumors analyzed (significance of difference medium vs. SCC supernatants, $P<0.05$). To confirm that MDSCs in SCCs produced NO, we isolated $CD11b^+$ cells using magnetic bead separation of collagenase-dispersed tumors. CD11b enrichment produced a fairly uniform population of $CD11b^+$ HLA-DR^{low} cells (Figure 3f) and analysis of the culture supernatants of these cells demonstrated that they produced NO (Figure 3g).

NO inhibits E-selectin expression by human dermal microvascular endothelial cells *in vitro*

NO inhibits the expression of endothelial adhesion receptors on human umbilical vein endothelial cells (HUVECs) and

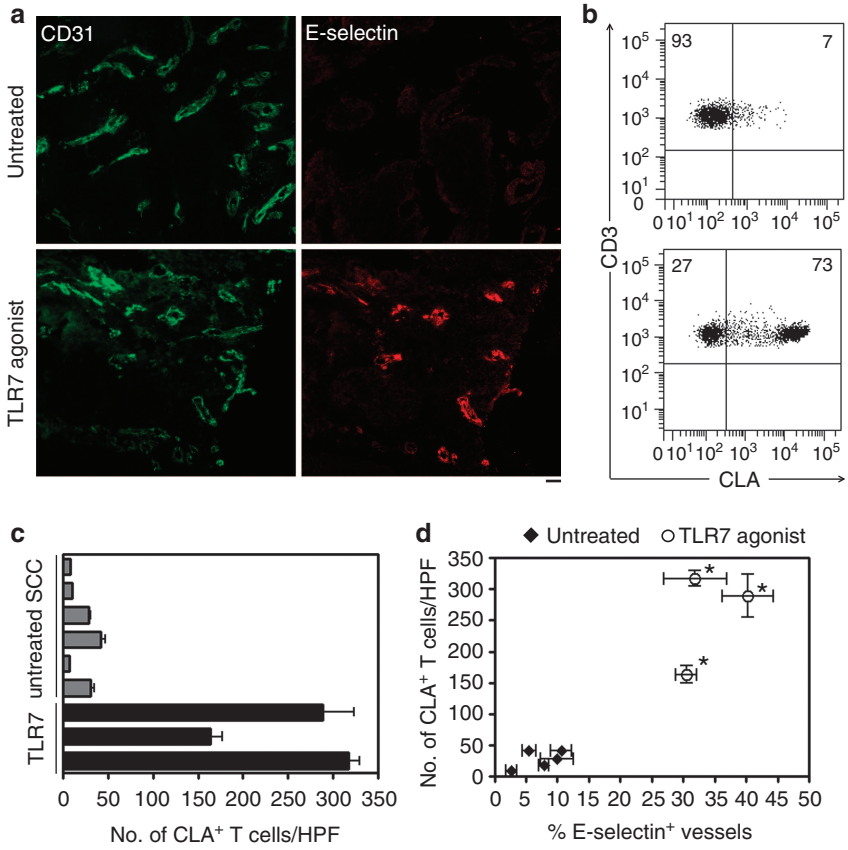

Figure 1. E-selectin expression on tumor vasculature correlates quantitatively with infiltration by CLA$^+$ T cells. (**a**) Untreated human squamous cell carcinomas (SCCs) lacked vascular expression of the skin T-cell-homing addressin E-selectin. Cryosections of tumor were costained for CD31 (a blood vessel marker, left panels) and E-selectin (right panels). SCCs treated with the Toll-like receptor 7 (TLR7) agonist imiquimod showed upregulation of E-selectin expression on tumor vessels (lower panels). Similar findings have been demonstrated in a total of 12 untreated tumors and six imiquimod-treated tumors. Scale bar = 100 μM. (**b**) CLA$^+$ T cells are excluded from untreated human SCCs but are present in imiquimod-treated tumors. Shown are T cells isolated from untreated (upper panel) and imiquimod-treated (lower panel) SCC tumors. (**c**) Exclusion of CLA$^+$ T cells from SCCs was reversed by topical treatment with TLR7 agonist before excision. The absolute numbers of CLA$^+$ T cells per high-power field (HPF) infiltrating untreated SCCs (gray bars) and TLR7 agonist-treated tumors (TLR7, black bars) are shown. (**d**) Vascular E-selectin expression correlated with the number of cutaneous lymphocyte antigen (CLA)-expressing T cells infiltrating SCCs and histologic evidence of tumor regression. The mean and SEM for both the number of CLA-expressing T cells and the % E-selectin positive vessels are shown. These studies demonstrate a strong correlation of vascular E-selectin expression with the ability of tumors to recruit CLA$^+$ skin-homing T cells. Tumors designated with an asterisk (*) had histologic evidence of tumor regression.

reduces adhesion of dendritic cells to endothelial monolayers *in vitro* (De Caterina *et al.*, 1995; De Palma *et al.*, 2006). Gene expression analyses have found significant differences between HUVECs and the microvascular endothelial cells found in tissues such as the skin (Chi *et al.*, 2003). We studied the effects of NO on human dermal microvascular endothelial cells (DMECs). To mimic the physiologic stimulation likely to occur within the tumor microenvironment, DMECs were co-cultured with TLR7 agonist-stimulated T-cell-depleted peripheral blood mononuclear cells antigen presenting cells (APCs). Under these conditions, NO potently inhibited endothelial E-selectin expression (Figure 4a and b). However, NO only partially inhibited E-selectin expression when endothelial cells were stimulated with 10 ng ml^{-1} of tumor necrosis factor-α (TNF-α). A similar biology was observed in HUVECs; NO completely inhibited E-selectin expression after physiologic stimulation with APCs but only partially inhibited expression after intense endothelial stimulation with TNF-α (Figure 4b).

MDSCs from SCCs suppress endothelial E-selectin expression *in vitro*

To determine if cell types in SCCs are capable of suppressing endothelial E-selectin expression, we cultured HUVECs in the presence of unfractionated cells from collagenase-dispersed SCC tumors. We observed a modest suppression of E-selectin under these conditions (Figure 4c). We then separated collagenase-dispersed SCCs into CD11b$^+$ and CD11b$^-$ fractions using magnetic bead separation (Figure 3f) and tested the ability of these cells to suppress endothelial E-selectin expression (Figure 4d and e). CD11b$^+$ cell fractions reproducibly inhibited endothelial E-selectin expression, whereas CD11b$^-$ fractions had no effect or modestly increased E-selectin expression.

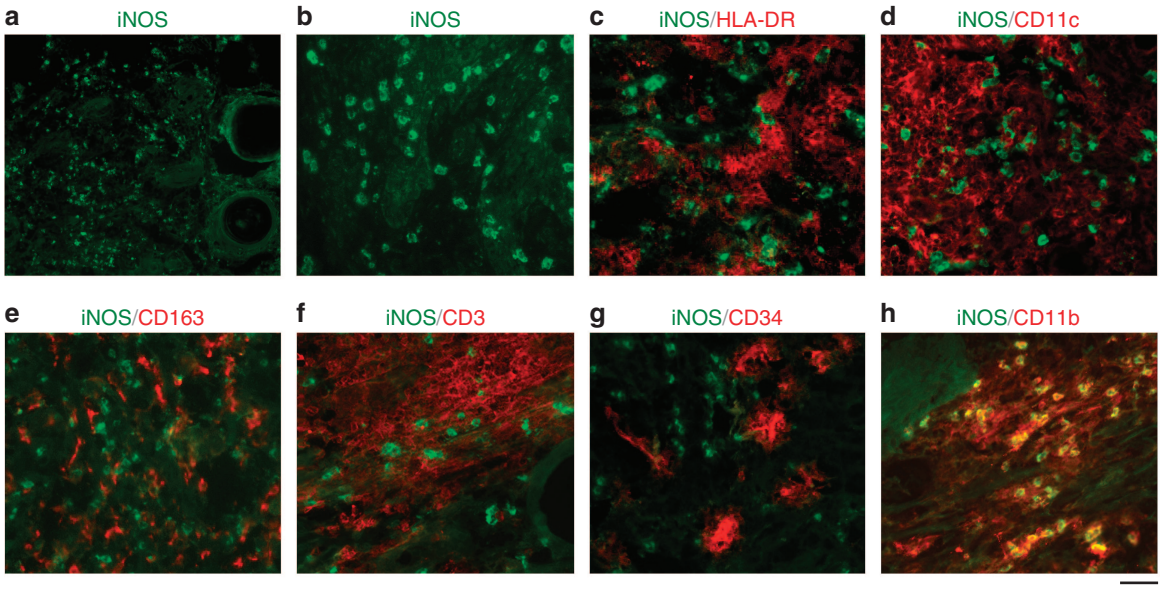

Figure 2. Inducible nitric oxide synthase–positive (iNOS$^+$) cells infiltrating squamous cell carcinoma (SCC) tumors lack HLA-DR and express CD11b, a phenotype consistent with myeloid-derived suppressor cells (MSDCs). (**a**, **b**) Costaining of SCC cryosections demonstrated that iNOS$^+$ cells were evident in SCC tumors. (**c**, **d**) iNOS$^+$ cells lacked expression of the dendritic cell markers HLA-DR and CD11c, (**e**) the macrophage marker CD163, (**f**) the T-cell marker CD3, and (**g**) the endothelial marker CD34. (**h**) iNOS$^+$ cells did express the myeloid marker CD11b. CD11b expression together with a lack of HLA-DR expression is a phenotype suggestive of MDSCs. Similar findings were observed in a total of eight SCC tumors. Scale bar = 100 μM.

MDSCs from SCCs express CCR2 and SCCs produce the CCR2 ligand HBD3

MDSCs from both mice and humans express the chemokine receptor CCR2 (chemokine (C-C motif) receptor 2) and CCL2 (chemokine (C-C motif) ligand 2) production by human tumors has been implicated in the migration of MDSCs into tumors (Huang *et al.*, 2007). MDSCs from collagenase-dispersed SCCs expressed CCR2 by flow cytometry analysis, whereas T cells from the same tumors did not (Figure 5a). Coimmunostaining for iNOS and CCR2 in tumor cryosections confirmed that iNOS$^+$ cells expressed CCR2 (Figure 5b). Quantitative reverse transcriptase–PCR analysis of SCC tumors showed that the CCL2 ligands CCL2, CCL7, CCL13, and human β-defensin 2 (HBD2) were expressed at comparable levels in normal skin and SCCs, whereas only the CCL2 ligand HBD3 was expressed at significantly higher levels in SCCs (Figure 5c). Immunostaining of SCC cryosections confirmed production of HBD3 in SCC tumors (Figure 5d).

iNOS inhibition induces SCC vascular E-selectin expression *in vitro*

To determine if NO production in tumors locally inhibits vascular E-selectin expression, we cultured portions of human SCCs for 24 hours in the iNOS inhibitor N_ω-nitro-L-arginine(L-NNA) in the presence or absence of TNF-α and then assayed for E-selectin expression by immunostaining of cryosections. We observed a marked upregulation of E-selectin expression in tumors treated with the iNOS inhibitor (Figure 6a and b). E-selectin was expressed on a mean 1.9% of blood vessels in SCCs treated with control medium ($n = 4$ tumors, SEM 0.58). After treatment with iNOS inhibitor, E-selectin was expressed by a mean 27.1% of tumor vessels ($n = 4$, SEM 1.06, control medium vs. iNOS inhibitor $P < 0.0001$). As expected, treatment of SCCs with TNF-α also increased expression of E-selectin (mean 19.6% positive vessels, SEM 2.17, $n = 4$, control medium vs. TNF-α treated $P = 0.0002$). Combining iNOS inhibition and treatment with TNF-α provided no additional increase in E-selectin expression above the use of iNOS inhibitors alone (iNOS inhibitor vs. TNF-α/iNOS inhibitor $P = 0.45$).

DISCUSSION

Impairment of T-cell homing is a major mechanism by which cutaneous SCCs evade immune responses. Vessels in SCC tumors lack expression of E-selectin and exclude the population of CLA$^+$ T cells thought to provide immune surveillance in the skin (Clark *et al.*, 2008). Induction of E-selectin expression on blood vessels by TLR7 agonist therapy leads to infiltration of the tumors by CLA$^+$ T cells producing IFN-γ, perforin, and granzyme, and is associated with histologic evidence of tumor regression (Clark *et al.*, 2008; Huang *et al.*, 2009). These clinical responses suggest that tumor-specific T cells exist within the circulation but cannot gain access to the tumor. Thus, the induction of appropriate T-cell homing addressins on tumor vessels has the potential to restore homing and potentially induce tumor destruction.

We report here that human SCCs are infiltrated by a population of NO-producing cells that express CD11b and CD33 and lack CD11c and HLA-DR, a phenotype suggestive of MDSCs (Diaz-Montero *et al.*, 2009; Gabrilovich and Nagaraj, 2009). MDSCs are a heterogenous population of myeloid cells that are enriched in the circulation of cancer patients as well as in many animal tumor models. MDSCs

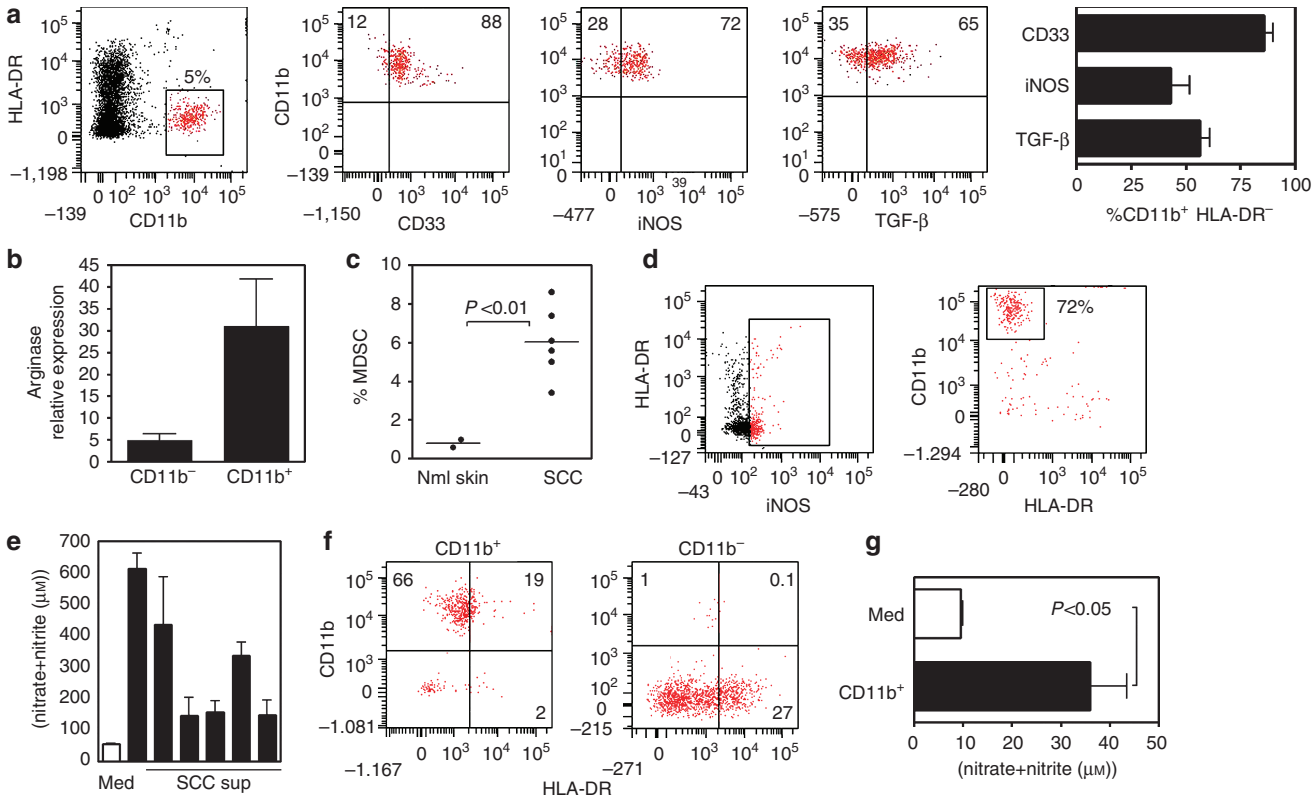

Figure 3. Myeloid-derived suppressor cells (MDSCs) are a major source of nitric oxide (NO) production in squamous cell carcinoma (SCC) tumors. (**a**) SCC tumors were dispersed by treatment with collagenase and the population of CD11b$^+$HLA-DR$^-$ cells was studied by flow cytometry. Most CD11b$^+$HLA-DR$^-$ cells expressed CD33, approximately half expressed inducible nitric oxide synthase (iNOS), and over half produced transforming growth factor-β (TGF-β). Representative histograms are shown and the mean and SEM of multiple donors are shown on the right ($n=5$ for CD33 and iNOS, $n=3$ for TGFβ). (**b**) Arginase I was increased in CD11b$^+$ cells isolated by magnetic bead separation from collagenase-dispersed tumors as assayed by quantative real-time PCR. The mean and SEM of three donors are shown. (**c**) In collagenase-dispersed tumors, a mean 6% of total tumor cells were CD11b$^+$ HLA-DR$^-$CD33$^+$ MDSCs but these cells were rare in normal skin (Nml skin). (**d**) In many tumors, MDSCs represented the majority of iNOS-expressing cells but non-MDSC cell types also expressed iNOS. (**e**) Analysis of SCC tumor supernatants using the Griess method demonstrated that NO was produced in SCC tumors. For each tumor, the mean and SEM of duplicate measurements of nitrate + nitrite are shown. (**f**, **g**) CD11b$^+$ MDSCs from SCCs produce NO. CD11b$^+$ MDSCs were enriched by magnetic bead separation from collagenase-treated tumors and cultured *in vitro*. Culture supernatants were analyzed for the presence of NO by the Griess method. The mean and SEM of measurements from three SCCs are shown.

play a major role in cancer-related immunosuppresssion and can potently suppress T-cell responses (Nagaraj *et al.*, 2010). The majority of CD11b$^+$CD11c$^-$HLA-DR$^-$ cells isolated from SCCs expressed iNOS, TGF-β, and arginase I, the three critical effector mechanisms used by MDSCs to suppress T-cell responses (Gabrilovich, 2004; Li *et al.*, 2009; Jia *et al.*, 2010). CD11b$^+$HLA-DR$^-$ cells comprised ~6% of total cells in SCCs, but were rare in normal skin (Figure 3c).

iNOS, a key enzyme that catalyzes NO production, is expressed in a variety of human cancers including malignant melanoma, breast, lung, prostate, and colorectal cancers (Lechner *et al.*, 2005). iNOS expression correlated with progression in human astrocytoma and prostate cancer, and patients with iNOS$^+$ melanomas had decreased survival (Lechner *et al.*, 2005; Ekmekcioglu *et al.*, 2006; Tanese *et al.*, 2011). NO has a variety of effects on immune cells including inhibition of T-cell activation, proliferation, and cytokine production (Bogdan, 2001), and animal studies suggest it may also reduce the adhesion of leukocytes to blood vessels. Inhibition of NO increased leukocyte rolling and adhesion in mesenteric venules in cats and rats and P-selectin expression was increased in rats after perfusion with NO inhibitors (Kubes *et al.*, 1991; Davenpeck *et al.*, 1994). In a mouse cancer model in which mouse mammary adenocarcinoma and human colon carcinoma cell lines were injected into the skin, treatment of animals with the iNOS inhibitor NG-nitro-L-arginine methyl ester led to increased rolling and stable adhesion of leukocytes to tumor vessels (Fukumura *et al.*, 1997).

We find that NO is produced by SCC tumors and that CD11b$^+$ MDSCs expressed iNOS and are significant sources of NO production within the SCC microenvironment. Small numbers of CD11b$^+$ HLA-DR$^{-/low}$ cells isolated from SCCs potently suppressed endothelial E-selectin expression, suggesting that even though MDSCs comprise a relatively small percentage of total tumor cells, they may play a critical role in downregulating vascular E-selectin and impairing T-cell trafficking into tumors. In a mouse model of B16 melanoma, MDSCs inhibited the migration of activated CD8 T cells into tumors but the mechanism of impaired T-cell homing was not identified (Lesokhin *et al.*, 2012). Our results suggest that NO

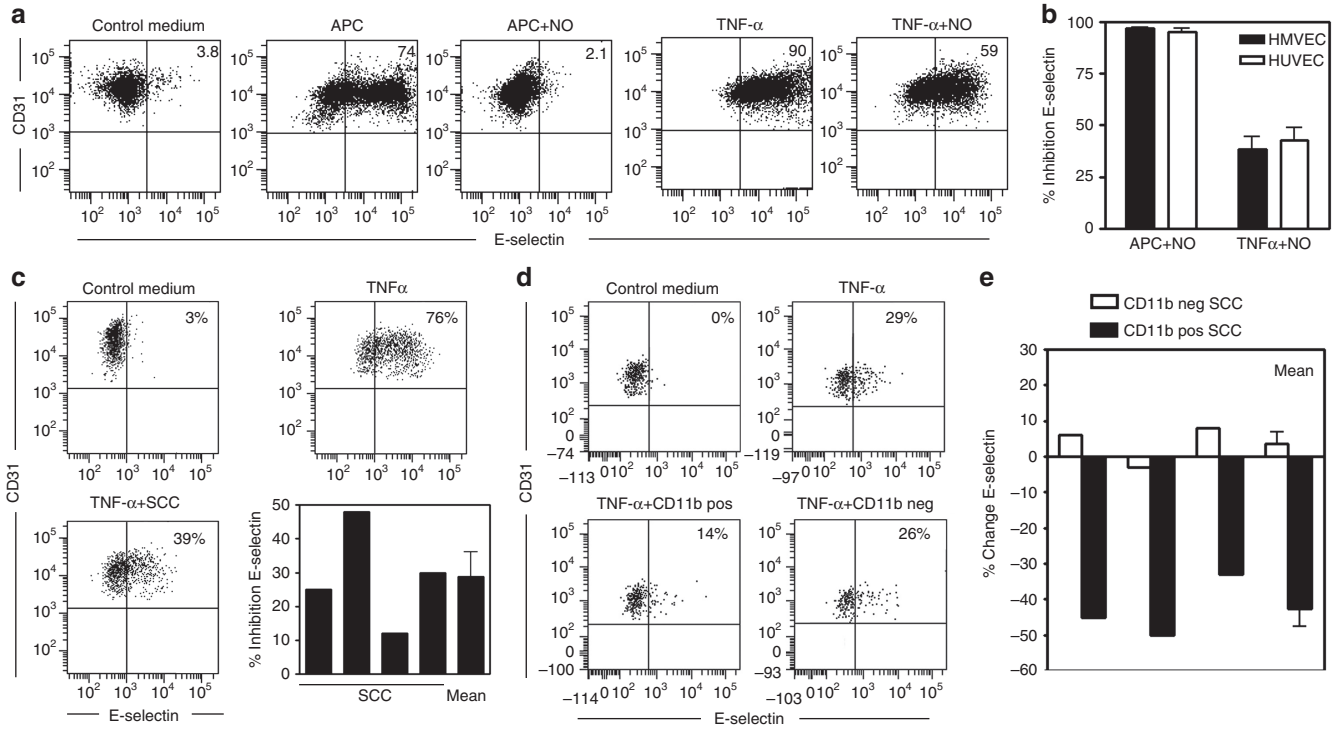

Figure 4. Nitric oxide (NO) and myeloid-derived suppressor cells (MDSCs) from squamous cell carcinomas (SCCs) inhibit human endothelial cell E-selectin expression. (**a**) Human skin dermal microvascular cells (DMECs) were stimulated with either Toll-like receptor 7 (TLR7) agonist activated APCs, mimicking physiologic stimulation, or the potent endothelial activator tumor necrosis factor-α (TNF-α) in the presence or absence of the NO donor spermine NONOate (NO). Treated cells were immunostained for E-selectin and analyzed by flow cytometry. NO potently suppressed E-selectin upregulation in response to stimulated APCs but only partially inhibited expression when endothelial cells were directly stimulated with tumor necrosis factor-α (TNF-α). (**b**) Percent inhibition of E-selectin expression by NO after stimulation with APCs or TNF-α. The mean and SEM of three different endothelial donors are shown. Human umbilical vein endothelial cells (HUVECs) responded similarly in that NO completely abrogated E-selectin expression in response to stimulated APCs (APC + NO) but only partially inhibited expression after stimulation with TNF-α. (**c**–**e**) MDSCs from SCCs suppress endothelial E-selectin expression *in vitro*. (**c**) HUVECs were stimulated with TNF-α in the presence or absence of collagenase-dispersed cells from SCC tumors. A modest but reproducible inhibition in E-selectin expression was observed. Representative histograms and individual results from four SCC tumors are shown, along with the mean and SEM of these measurements. (**d**, **e**) $CD11b^+$ MDSCs from SCC tumors suppress endothelial E-selectin expression. $CD11b^+$ and $CD11b^-$ cell populations were obtained by magnetic bead separation from collagenase-dispersed SCC tumors. HUVECs were stimulated with TNF-α in the presence or absence of $CD11b^+$ and $CD11b^-$ cells. (**d**) Representative histograms and (**e**) individual results from three SCC tumors are shown, along with the mean and SEM of these measurements. $CD11b^+$ MDSCs reproducibly inhibited expression of endothelial E-selectin. APC, antigen presenting cell.

produced by MDSCs inhibits vascular E-selectin, likely impairing T-cell migration in to tumors, and that this may be another mechanism by which MDSCs impair antitumor immunity.

Human studies have been limited but *in vitro* treatment of HUVECs with NO has been shown to reduce addressin expression and dendritic cell adhesion (De Caterina *et al.*, 1995; De Palma *et al.*, 2006). We found that NO completely abrogated the expression of E-selectin when DMECs were physiologically stimulated with activated APCs, but could only partially inhibit E-selectin expression when endothelial cells were strongly and directly stimulated with 10 ng ml^{-1} of TNF-α (Figure 4a and b). It may therefore be possible to overcome impaired E-selectin expression by either inhibiting iNOS activity or by potently and directly stimulating endothelial cells. We found that HUVECs and DMECs derived from human skin responded very similarly to NO, suggesting that HUVECs, which are easier to obtain and grow, may be useful in drug screens used to identify new agents that activate endothelial cells.

MDSCs in both humans and animal models express the chemokine receptor CCR2 (Huang *et al.*, 2007; Lesokhin *et al.*, 2012). CCL2 is produced by human breast, gastric, and ovarian cancers and inhibition of CCR2/CCL2 signaling in mouse cancer models reduced MDSC migration and MDSC-induced tumor cell growth (Huang *et al.*, 2007). In addition to their antimicrobial function, HBD2 and HBD3 can induce cell chemotaxis via CCR2 (Rohrl *et al.*, 2010). HBD3/CCR2 interactions promoted migration of macrophages into tumors in a mouse model of oral carcinoma (Jin *et al.*, 2010). We found that $iNOS^+$ MDSCs in SCC tumors expressed CCR2, and SCC tumors expressed the CCR2 ligand HBD3, suggesting that CCR2/HBD3 interactions may play a role in the recruitment of MDSCs to tumors (Figure 5).

Last, we tested the ability of iNOS inhibitors and TNF-α to restore E-selectin expression in freshly excised human SCC tumors. iNOS inhibition alone markedly enhanced E-selectin expression on tumor vessels (Figure 6). Remarkably, iNOS inhibition alone induced E-selectin expression at similar or greater levels than TNF-α. Moreover, E-selectin was

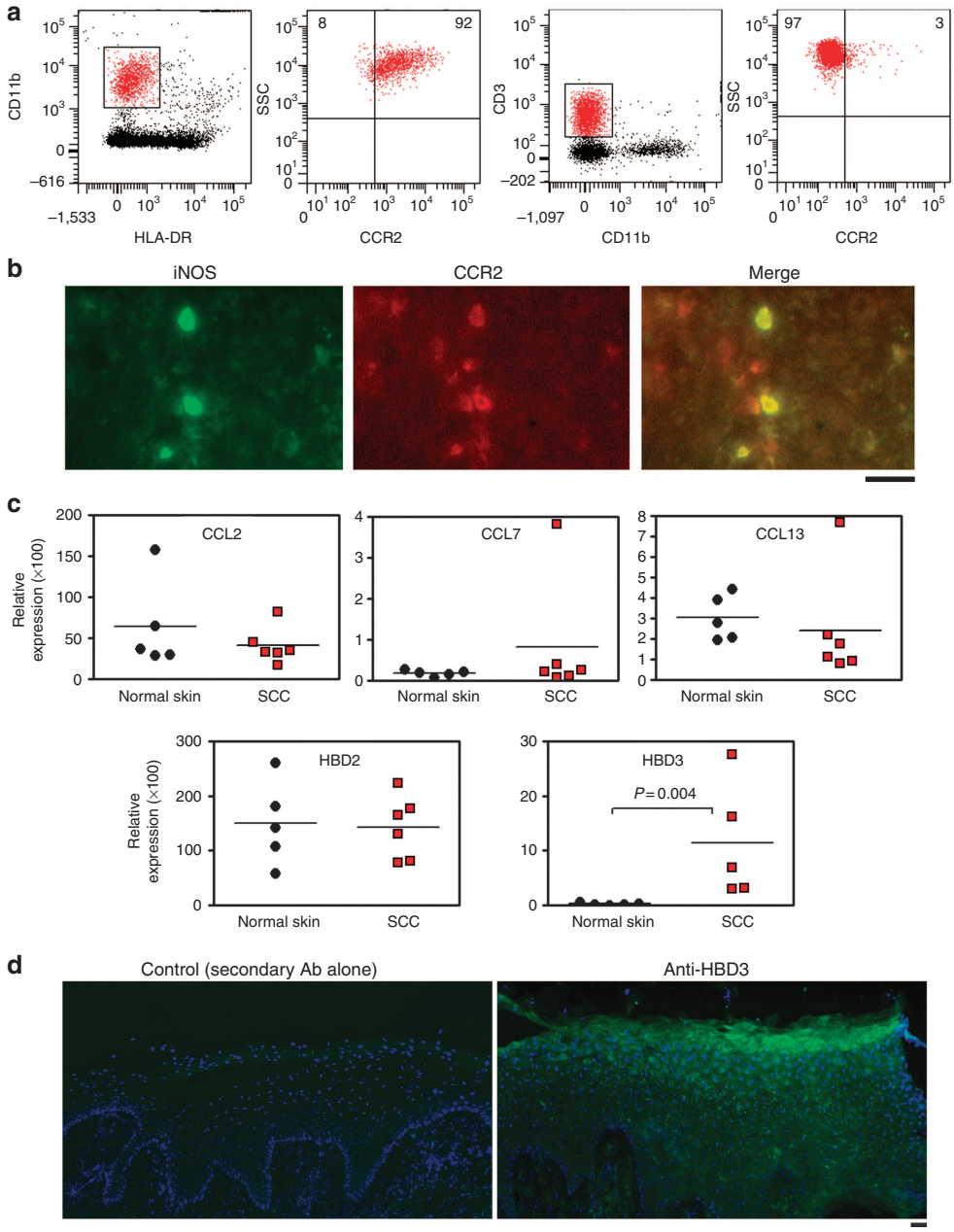

Figure 5. Myeloid-derived suppressor cells (MDSCs) from squamous cell carcinomas (SCCs) express CCR2 (chemokine (C-C motif) receptor 2) and SCCs produce the CCR2 ligand human β-defensin 3 (HBD3). (**a**) $CD11b^+HLA\text{-}DR^-$ MDSCs from collagenase-dispersed SCCs uniformly expressed CCR2, whereas $CD3^+$ T cells lacked CCR2 expression. (**b**) Immunostaining of SCC cryosections confirmed that inducible nitric oxide synthase–positive ($iNOS^+$) cells in SCC tumors coexpress CCR2. (**c**) Quantitative real-time PCR (RT-PCR) analysis of SCC tumors and normal human skin demonstrated selective production of the CCR2 ligand HBD3 in SCCs. (**d**) Immunostaining of SCC cryosections confirmed production of HBD3 by SCC tumor cells. Scale bar = 100 μM.

expressed at levels comparable to those observed in SCCs undergoing immunologic destruction after topical treatment with TLR7 agonist (Figures 1d and 6b). SCCs undergoing immunologic destruction after TLR7 agonist therapy expressed E-selectin on a mean 34.2% of blood vessels as compared with 27.2% of blood vessels following *in vitro* treatment with iNOS inhibitor ($P > 0.05$). Although the limitations of working with humans preclude us from directly demonstrating that T-cell recruitment into SCCs is enhanced as a result of iNOS inhibition, these studies strongly suggest that iNOS inhibition induces vascular E-selectin at levels capable of enhancing T-cell entry into tumors. To our knowledge, it is previously unreported in a human cancer that iNOS activity impairs the expression of vascular addressins critical for T-cell recruitment.

Our results suggest that treatment with iNOS inhibitors or potent stimulators of endothelial activation both have the potential to restore addressin expression in SCCs without the attendant widespread immune activation observed with TLR agonists such as imiquimod. A topically applied inhibitor of

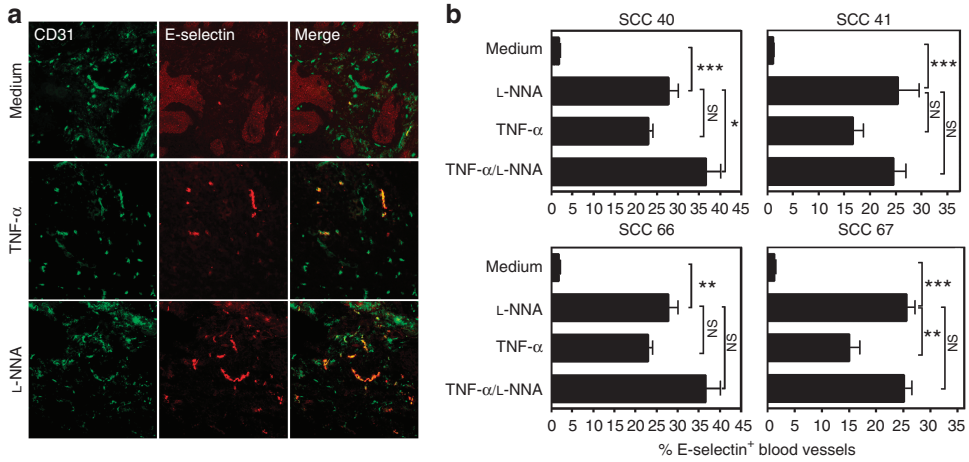

Figure 6. Inhibition of inducible nitric oxide synthase (iNOS) activity restores vascular E-selectin expression in squamous cell carcinoma (SCC) tumors. (**a**) *In vitro* treatment of human SCCs induced vascular E-selectin expression. SCC tumors were cultured for 24 hours in medium alone, tumor necrosis factor-α (TNF-α), or with the iNOS inhibitor N_{ω}-nitro-L-arginine (L-NNA). Tumors were then cryosectioned and stained for vascular E-selectin expression. SCCs treated with TNF-α or iNOS inhibitor showed induction of vascular E-selectin expression on a subset of vessels. (**b**) iNOS inhibition potently induced vascular E-selectin expression. The mean and SEM of the % E-selectin expressing vessels in 10 high-power fields are shown. *In vitro* treatment of tumors with iNOS inhibitor induced vascular E-selectin at levels comparable to or greater than that observed after treatment with TNF-α. Results from four SCC tumors are shown; comparable results were observed in seven additional SCCs. NS, not significant. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.0005$. Scale bar = 500 μM.

iNOS is currently in clinical trials for the treatment of neuropathic pain (LaBuda *et al.*, 2006). Because immune evasion in SCCs is primarily an issue of impaired T-cell homing, our studies suggest that topically applied iNOS inhibitors or potent endothelial activating agents may be effective, either alone or in combination with other therapies, in the treatment of SCCs and their premalignant precursor lesions, actinic keratoses.

It is becoming increasingly appreciated that cancer destruction requires not only the generation of tumor-specific T cells, but also the ability of these T cells to access the tumor once they are generated (Gajewski, 2007). Impaired T-cell homing as a result of decreased vascular addressin expression has been reported in a number of human cancers, including malignant melanoma, breast, gastric, and lung cancers (Piali *et al.*, 1995; Madhavan *et al.*, 2002; Weishaupt *et al.*, 2007). Melanoma metastases express low levels of the addressins E-selectin, P-selectin, and ICAM-1, and this is associated with low numbers of T cells within the metastatic tumor nodules (Weishaupt *et al.*, 2007). Our work suggests that local production of NO within tumors could be a common mechanism for impaired T-cell homing. If this proves to be the case, iNOS inhibition used in concert with agents that enhance the presentation of tumor antigens have the potential to enhance immune responses to many human cancers.

MATERIALS AND METHODS

SCC samples

Tumor samples consisted of tumors removed before taking the first Moh's section during Moh's micrographic excision of biopsy-proven squamous cell carcinomas removed from immunocompetent individuals. Acquisition of tumor samples and all studies were approved by the Partners Institutional Review Board and were performed in accordance with the Declaration of Helsinki Principles. Because this work utilized discarded tissues with no identifiable personal information, the partners IRB ruled that no informed written patient consent was required.

Immunofluorescence studies

Sections (5 μm) were cut from SCC blocks frozen in optimal cutting temperature compound. They were fixed in acetone, air dried, rehydrated in phosphate-buffered saline, and blocked with human IgG (Jackson Immunoresearch, West Grove, PA). Sections were incubated with a biotinylated anti-E-selectin (clone 68-5H11, BD) at 5 μg ml^{-1}, rinsed in phosphate-buffered saline/1% BSA, and costained with streptavidin/phycoerythrin (1 μg ml^{-1}, R&D Systems, Minneapolis, MN) and CD31 FITC (1:40 clone WM59, BD, Franklin Lakes, NJ). Sections were rinsed and mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (Invitrogen, Grand Island, NY). E-selectin^{+} blood vessels were enumerated in ten ×100 HPFs. To quantify CLA^{+} T cells, sections were costained with phycoerythrin anti-CD3 (1:40, BD) and FITC anti-CLA (1:25, BD). The number of CLA^{+} infiltrating T cells was determined by counting CLA^{+} cells directly in 10 HPFs or by counting total T cells in 10 HPFs and multiplying by the % CLA^{+} T cells of total T cells obtained from 5 HPFs. For examination of iNOS-expressing cells, sections were costained with directly conjugated mAbs (BD) and FITC anti-iNOS (1:20, BD). For HBD3 staining, sections were stained with rabbit anti human HBD3 mAb (1:00, FL-67 Santa Cruz Biotech, Santa Cruz, CA) followed by Alexa Fluor 488 goat anti rabbit polyclonal antibody (1:100, Invitrogen). In all studies, 4,6-diamidino-2-phenylindole nuclear stain was used to confirm the presence of invasive tumor as determined by the presence of large atypical keratinocyte nuclei. Sections were photographed using a Nikon Eclipse 6600 microscope equipped with Nikon Plan Fluor objective lenses, Nikon (Melville, NY). Images were captured with a SPOT RT model 2.3.1 camera (Diagnostic Instruments, Sterling Heights, MI) and were acquired with SPOT 4.0.9 software (Diagnostic Instruments).

Isolation and flow cytometry analysis of T cells and MDSCs from SCC tumors

For isolation of MDSCs from SCCs, tumors were minced and dissociated with 0.2% type I collagenase (Invitrogen) and 30 Kunitz Units ml^{-1} of DNAse (Sigma, St Louis, MO) for 2 hours at 37 °C with vigorous shaking. $CD11b^+$ cells were isolated from collagenase-treated tumors by staining with anti-CD11b-phycoerythrin mAb (R&D systems) and anti-phycoerythrin micro-beads (Miltenyi Biotech, Auburn, CA) followed by AutoMACS separation (Miltenyi Biotech). For TGF-β staining, cells were stimulated overnight with 1 μg ml^{-1} lipopolysaccharide from *E. coli* (Sigma) and 100 IU ml^{-1} IFN-γ (R&D systems) to activate MDSCs (Greifenberg *et al.*, 2009). Flow cytometry analysis was performed using directly conjugated mAbs from BD. Analysis of flow cytometry samples was performed on a Becton Dickinson FACSCanto instrument and data were analyzed using FACSDiva software (BD).

Assay of SCC supernatants and $CD11b^+$ cells for NO production

For analysis of SCC supernatants, 3 mm^3 tumor fragments were cultured for 24 hours in Iscoves medium supplemented with 10% human AB serum, fungizone, gentamicin, penicillin/streptomycin, L-glutamine and 0.6 mM L-arginine. Supernatants were concentrated using Microcon centrifugal filter devices (Millipore, Billerica, MA). For analysis of NO production from SCC $CD11b^+$ cells, $CD11b^+$ cells were isolated from collagenase-treated SCCs by magnetic bead separation as described and 10,000 $CD11b^+$ cells were incubated for 12 hours with 1 μg ml^{-1} *E. coli* lipopolysaccharide and 100 IU ml^{-1} IFN-γ. Supernatants were assayed for NO using the QuantiChrom Nitric Oxide assay kit or the Parameter Total NO/Nitrite/Nitrate Assay Kit (R&D Systems), as per the manufacturer's instructions.

Endothelial cell studies

Human DMECs and HUVECs (Lonza, Walkersville, MD) were cultured in EGM-2 bulletkit growth medium (Lonza). Endothelial cells at 90% confluency were stimulated for 12 hours with activated APCs or 10 ng ml^{-1} TNF-α (R&D systems) with or without 0.5 mM NO donor spermine NONOate (Sigma). Human peripheral blood APCs were isolated from products discarded following plasmapheresis of healthy donors by density centrifugation using histopaque-1077 (Sigma) and depletion of T cells using the Pan-T isolation kit and AutoMACS instrument (Miltenyi Biotech). APCs (2.5×10^6) and 3 μM imiquimod (added to stimulate APCs) were added to wells containing cultured endothelial cells for 12 hours. Endothelial cells were then stained with directly conjugated antibodies to CD31 and E-selectin (BD) and acquired on a BD FACSCanto flow cytometer. Data were analyzed using FACSDiva software (V6.1). For experiments involving cocultures of endothelial cells with cells from SCC tumors, cells obtained from collagenase-treated SCCs were rested overnight at in complete Iscove's medium in the presence of 10 ng ml^{-1} GM-CSF (R&D systems) to maintain myeloid cell viability (Ko *et al.*, 2009). Cells from SCCs were added to endothelial cells in a 1:1 ratio and stimulated with 1 ng ml^{-1} TNF-α (R&D systems). In a second group of experiments, 5,000 AutoMACS-enriched $CD11b^+$ cells from collagenase-treated SCCs were added to wells containing 5,000 endothelial cells stimulated with TNF-α 1 ng ml^{-1}; immunostaining of endothelial cells was performed 12 hours later as described.

Quantitative real-time PCR for CCR2 ligands and arginase I

Total RNA was isolated from cryosections of SCCs and normal skin or from cells isolated from SCC tumors using the RNeasy Lipid Tissue kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Complementary DNA was generated using the Quanti-Tect reverse transcription kit (Qiagen) and quantitative real-time PCR was performed using the ABI StepONE plus instrument and the Fast SYBR green master mix (Applied Biosystems, Carlsbad, CA). Expression of each ligand transcript was determined relative to the reference gene transcript, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and calculated as $2^{\wedge}-(C_{t,\ ligand}-C_{t,\ GAPDH})$. The primers used to detect the ligands and the reference transcripts were purchased from Origene Technologies (Rockville, MD) and were as follows: hBD2 (F-5′-GGCGTAGAAGTTCTCTGTCTCC-3′; R-5′-GAAGCAGGAGAAAAGGATGGAG-3′), hBD3 (F-5′-GGTGAAG CCTAGCAGCTATGAG-3′; R-5′-GCCGCCTCTGACTCTGCAATA-3′), CCL2 (F-5′-AGAATCACCAGCAGCAAGTGTCC-3′, R-5′-TCCTGAA CCCACTTCTGCTTGG-3′), CCL7 (F-5′-ACAGAAGGACCACCAGTAG CCA-3′; R-5′-GGTGCTTCATAAAGTCCTGGACC-3′), CCL13 (F-5′-GA TCTCCTTGCAGAGGCTGAAG-3′; R-5′-TCTGGACCCACTTCTCCTTT GG-3′), and GAPDH (F-5′-GAGTCAACGGATTTGGTCGT-3′; R-5′-CAT GGGTGGAATCATATTGGA-3′).

In vitro treatment of SCCs with iNOS inhibitor and TNF-α

SCCs were cultured for 24 hours in control medium (Iscoves, 10% human AB serum, fungizone, gentamicin, penicillin/streptomycin, L-glutamine, 0.6 mM L-arginine) in the presence or absence of 0.6 mM iNOS inhibitor L-NNA (Sigma) and/or 10 ng ml^{-1} TNF-α (R&D), then embedded in optimal cutting temperature compound, cryosectioned and stained for CD31 and E-selectin as described.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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